BAC to immunology – bacterial artificial chromosome-mediated transgenesis for targeting of immune cells

Tim Sparwasser[1] and Gérard Eberl[2]

[1]*Institut für Medizinische Mikrobiologie, Immunologie & Hygiene, Technische Universität München, Munich, Germany, and* [2]*Laboratory of Lymphoid Tissue Development, Institut Pasteur, Paris, France*

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Correspondence: Dr Tim Sparwasser, Institut für Medizinische Mikrobiologie, Immunologie & Hygiene, Technische Universität München, Trogerstrasse 30, 81675 Munich, Germany.
Email: tim.sparwasser@lrz.tum.de or
Dr Gérard Eberl, Laboratory of Lymphoid Tissue Development, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris, France.
Email: geberl@pasteur.fr
Senior author: Dr Tim Sparwasser

Summary

Thirty years after the first transgenic mouse was produced, a plethora of genetic tools has been developed to study immune cells *in vivo*. A powerful development is the bacterial artificial chromosome (BAC) transgenic approach, combining advantages of both conventional transgenic and knock-in gene-targeting strategies. In immunology the potential of BAC transgenic technology has yet to be fully harvested and, combined with a variety of elegant genetic tools, it will allow the analysis of complex immunological processes *in vivo*. In this short review, we discuss the applications of BACs in immunology, such as identification of regulatory regions, expression and cell-fate mapping, cell ablation, conditional mutations and the generation of humanized mice.

Keywords: bacterial artificial chromosome; mouse model; transgenic

Introduction

In 1976 Jaenisch *et al*. were the first to generate transgenic mice by introducing viral genomic DNA from Moloney leukaemia virus into the germline of mice.[1] Most of the recent advances in understanding the immune system owe their existence to the development of the gene-targeting techniques established in the late 1980s.[2,3] As of now, approximately 10% of all murine genes have been genetically targeted[4] and ambitious projects, such as the Knock-Out Mouse Project (KOMP)[4] and the European Conditional Mouse Mutagenesis (EUCOMM) programme[5] aim to mutate every individual murine gene. Recent developments of phage artificial chromosomes (PACs),[6,7] yeast artificial chromosomes (YACs)[8] and, in particular, F factor-based bacterial artificial chromosomes (BACs)[9,10] have made genomic fragments spanning several hundred kilobases (kb) from humans, mice and many other species available through public genomic databases. As a result, the tedious screening for genomic DNA clones in complex libraries is reduced to the selection, *in silico*, of a PAC, YAC or BAC clone corresponding to the locus of interest. BACs, on average 100–300 kb in size, have been the templates for most genome sequence projects, and, as a consequence, are mapped in their thousands to the human and mouse genomes. Given their large size, BACs encode most, if not all, regulatory regions of a gene, as well as the *cis*-elements that define expression domains, such as scaffold/matrix attachment regions,[11] and isolate the gene from distal regulation. Using transgenesis technology, BACs have therefore been used as an economical surrogate to mouse gene-targeting (knock-in) technology, in which an allele is modified in stem cells before these are re-implanted into blastocysts. Here, we attempt to give a short review of the potentials of BAC transgene technology and the impact of BAC transgenic mice in basic and human immunology.

Abbreviations: BAC, bacterial artificial chromosome; DT, diphtheria toxin; DTR, diphtheria toxin receptor; GFP, green fluorescent protein; IRES, internal ribosome entry site; PAC, phage artificial chromosome; RAG, recombinase activity gene; Tregs, regulatory T cells; YAC, yeast artificial chromosome; YFP, yellow fluorescent protein.

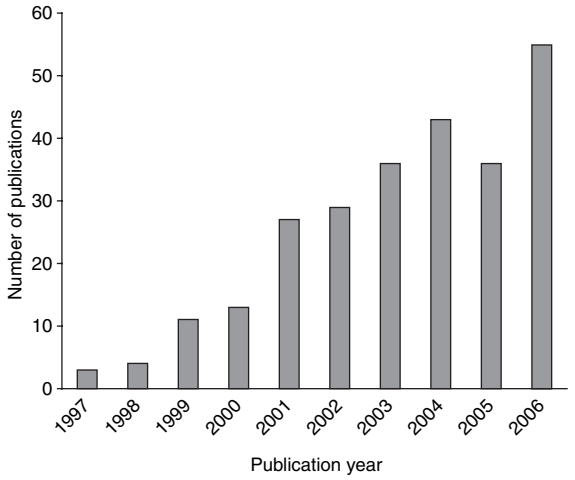

Figure 1. The graph shows the approximate number of papers published every year on BAC transgenic mice since their original description in 1997, based on a *PubMed* search using the terms 'transgenic and (mice OR mouse OR murine) and (BAC OR bacterial artificial chromosome) and 1996–2006 [Publication Date].

BAC transgenic mice

Heintz and colleagues were the first to report a technique for the modification of BACs. This method allowed the generation of BAC transgenic mice for gene expression mapping using the green fluorescent protein reporter GFP.[12] Several additional protocols have been developed subsequently for the modification of BACs in bacteria and the generation of BAC transgenic mice.[13–16] Ambitious projects have emerged based on BAC transgenesis, which aim at mapping all genes expressed in the central nervous system (GENSAT project[17]), or characterizing potential targets for drug development (VelociGene, Regeneron[18]), resulting in a boom in the number of BAC transgenic mice (Fig. 1).

The different techniques used for the modification of BACs rely on homologous recombination between cloned or synthesized genomic fragments and the cognate BAC locus, resulting in the insertion of heterologous sequences or the deletion of endogenous sequences. The first method, originally developed by Heintz and co-workers,[12,14] is based on bacterial recombinase A for the recombination of homologous fragments of approximately 1 kb, whereas the method developed by Stewart and colleagues[13] is based on λ phage DNA repair enzymes to induce recombination of homologous fragments as short as 50 nucleotides. In addition, random modification of BACs by the integration of reporter genes has been achieved by using bacterial transposases.[19] In essence, BAC transgenesis combines the advantages of conventional transgenic approaches (speed, ease of use) with those of standard knock-in techniques (use of uncharacterized promoters or complete regulons). After modification of target genes *in vitro*, BACs are purified and injected into fertilized eggs that are subsequently implanted into the oviducts of foster mothers (the advantages and pitfalls of the three approaches are compared in Table 1). Diverse and complex genetic strategies can be applied using modified BACs because of the rapidity of the DNA modification protocols that are carried out in bacteria.

BAC transgenic mice in basic immunology

The development of BAC technology offered new possibilities for transgenic model systems in basic immunology that are discussed below and summarized in Fig. 2.

Expression mapping and identification of murine regulatory regions

Identifying regulatory regions and studying gene expression can be challenging, in particular if the genes of interest cannot be detected by antibody-mediated staining. Insertion of a reporter gene into the locus of interest, coupled to serial deletions, allows expression mapping as well as subsequent analysis of putative regulatory regions.

One of the first examples using this approach was reported by Nussenzweig and co-workers.[20] The aim of the study was to elucidate the genetic regulation of recombinase activating genes 1 (*RAG1*) and *RAG2* expression. Yu *et al.* generated several lines of BAC transgenic mice that expressed *GFP* instead of *RAG2* and/or yellow fluorescent protein (*YFP*) gene instead of *RAG1* by replacing exons at the start codon.[20] *In vitro* recombined BACs of different lengths were selected to cover different 5′ and 3′ regions expected to contain regulatory elements. Upon BAC transgenesis it could be demonstrated that the 5′ region of the *RAG1* gene contained basal promoter activity, but was not sufficient for high *RAG1-YFP* gene expression in double-positive thymocytes. Both *RAG1* and *RAG2* appeared to be co-ordinately regulated by a distal locus control region located 5′ of the *RAG2* gene.[20]

BAC technology has been frequently used in the subsequent years for the study of regulatory regions, including the identification of a T-cell enhancer,[21] deletion of a T-cell receptor β enhancer,[22] mutation of a signal transducer and activator of transcription 6 (STAT6) binding site and additional mutation in putative regulatory regions of a rearranged V(D)J gene or the entire immunoglobulin heavy chain constant region locus.[19,23,24] Finally, by using reporter BACs of different lengths, Lee *et al.* could demonstrate that a 25-kb region within the 3′ area of the T helper type 2 locus encoding interleukin-4 (IL-4), IL-5 and IL-13, the associated *RAD50* gene contained locus control region activity.[19]

Table 1. The generation of mutant mice using BAC technology, short transgenes or stem cell technology

	BAC transgenes	Short transgenes	Knock-in
Time for generation	**3–6 months**[1]	**3–6 months**	*1–2 years*
Technical difficulty	Medium	**Low**	*High*
Copy number	**Usually low (< 10)**	Usually high (> 10)	**1–2**
Promoter knowledge	**Not required**	*Required*[2]	**Not required**
Genomic insertion site	Not determined[3]	*Not determined*[3]	**Determined**
Chromatin interference	**Low probability**[4]	High probability[4]	**Low probability**
Structural integrity	Difficult to assess[5]	**Easy to assess**	**Easy to assess**
Faithful expression	**High probability**	Low probability	**High probability**
Bystander transgenes	High probability[6]	**No**	**No**

[1]Code for the technical characteristics of the mutation strategies: advantage (bold type), potential disadvantage (normal type), disadvantage (italic type).
[2]The short transgene requires a synthetic or a minimal promoter that does not exceed a few kilobases in length.
[3]The transgene integration site has less or no impact on BACs, contrary to most short transgenes, as BACs have a high probability of carrying locus control regions. Nevertheless, short transgenes can be knocked into a determined locus in embryonic stem cells to avoid chromatin interference and therefore increase the probability of faithful expression.[43]
[4]The local chromatin can exert dominant regulation over the transgene through the presence of strong promoters, silencers, or locus control regions. In addition, the repetitive structure of transgene concatemers may induce epigenetic silencing through methylation.
[5]The structural integrity of the BAC transgene is difficult to assess, as the size of the BAC can exceed 200 kb. However, before injection of the BAC into the pronucleus of fertilized eggs, the BAC integrity can be assessed by pulsed-field gel electrophoresis.
[6]Because of their large size, BACs usually carry genes in addition the gene of interest, creating polyploidies upon transgenesis. However, only the gene of interest will be engineered to drive a reporter or an effector gene, and bystander genes may be deleted if polyploidy has an impact.

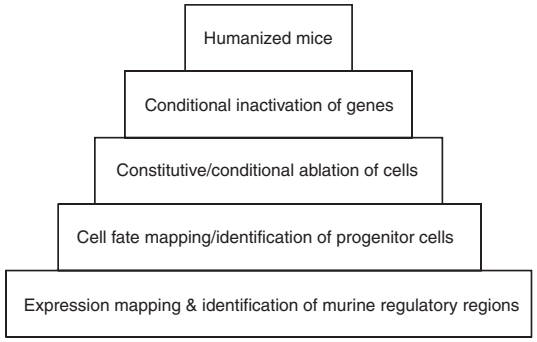

Figure 2. The graph shows possible transgenic model systems in immunology using bacterial artificial chromosome technology.

Cell-fate mapping

Cell-fate mapping aims to label a population of cells and identify its progeny. In immunology, the traditional approach for cell-fate mapping of haematopoietic progenitors has been the adoptive transfer of cells into irradiated animals.[25,26] However, even though this approach allows the determination of whether a particular progenitor can differentiate into a specific population of cells, it does not assess whether this pathway is effective in a normal, non-lymphopenic animal. Genetic cell fate mapping has been performed in many instances in developmental biology, but rarely in immunology, such as for the identification of memory $CD8^+$ T cells.[27]

Recently, the progeny of cells expressing the hormone nuclear receptor gene retinoic acid-related orphan receptor (*Rorc(γt)*) has been determined using BAC technology.[28,29] *Rorc(γt)* is expressed in fetal lymphoid tissue inducer cells required for the development of lymph nodes and Peyer's patches and immature thymocytes. It is also expressed in the adult gut in cells similar to fetal lymphoid tissue inducer cells that form small clusters, the cryptopatches. These cryptopatches have been suggested to be the site for extrathymic generation of T cells[30] but genetic cell fate mapping demonstrated that this pathway is not efficient in normal mice, i.e. mice that have a normal haematopoietic compartment. Rather, these studies showed that most if not all T-cell receptor $\alpha\beta^+$ cells were derived from *RORγt*-expressing immature thymocytes.

Ablation of cells

Gene-deficient mice allow the inference of the function of a gene, and have been a fundamental tool for immunologists. Another strategy consists of the ablation of cells expressing a particular gene that confers sensitivity to an injectable toxin on murine immune cells. This method

has been reported recently in CD11c-DTR conventional transgenic mice, where expression of the primate diphtheria toxin receptor (DTR) in mouse dendritic cells allows their specific and inducible depletion upon administration of diphtheria toxin (DT).[31]

We have used a similar strategy, in conjunction with BAC technology, to induce specific ablation of natural regulatory T cells (Tregs)[32]. Tregs are essential regulators of peripheral tolerance[33] and immunity[34] but the mechanism of tolerance induction remains unclear, mostly because of a lack of suitable genetic mouse models. Murine Tregs express specifically the forkhead transcription factor Foxp3.[33] We have modified a BAC coding for Foxp3 by insertion of a *DTR-GFP* reporter gene in exon I. BAC transgenic DEREG mice (for DEpletion of REGulatory T cells) show high expression of the DTR-GFP fusion protein in $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs that can be depleted by injection of DT.[32] Expression of the transgene allows both tracking (GFP) and ablation (DT) of Tregs at any time-point during infection, allergy or autoimmunity. In contrast to the common depletion protocols using anti-CD25 antibodies, DEREG mice grant highly specific depletion of Tregs, and therefore may provide insights into the mechanism of action during different phases of the immune response.

Kaplan *et al.* directly expressed the toxic subunit of DT in the target cells.[35] In this case, the human Langerin promoter was used, and the last coding exon on the BAC was modified by insertion of an IRES-DTA sequence. The human Langerin locus provided very specific expression of the transgene in Langerhans cells but not in any other dendritic cell subset as reported for the murine orthologue. The additional advantage of the human BAC in this model was that antibodies specific for human Langerin could be used to monitor complete ablation of epidermal Langerhans cells.

Conditional mutants

Conditional knock-out mice carry a tissue-specific gene deficiency. This procedure requires two modifications of the mouse genome. The first is usually present as a transgene and controls tissue-specific expression of the λ phage Cre recombinase, and the second is an insertion into the endogenous mouse locus of *loxP* sites flanking exons or regulatory regions of the gene to be knocked out. Whereas the second modification has to be achieved by recombination in embryonic stem cells, tissue-specific Cre expression can be generated by BAC transgenic mice. This option has been recently applied specifically to knock-out the anti-apoptotic factor Bcl-xL in immature thymocytes expressing the nuclear hormone receptor RORγt[29] to demonstrate the role of this factor in the survival of double-positive thymocytes.

Another option comprises the complementation of gene knock-out mice with the same gene under tissue-specific control. This experimental system has been used with BACs to show that the immunoglobulin β-chain is required for the survival of specific B-cell precursor populations and to mediate B-cell differentiation.[36]

Humanized mice

The original finding that a genomic DNA fragment containing the chicken lysozyme gene locus could induce expression of chicken lysozyme RNA specifically and at high levels in mouse macrophages, thus replicating the expression pattern in chicken, indicated that regulon transfer was possible across species.[37] In fact, several experiments carried out in the 1990s with YACs paved the way for the use of large fragments of human genomic DNA for the generation of humanized transgenic mice. Humanized mice are important for the expression and study of immunological effectors, the identification of genes involved in human pathologies, and the creation of novel disease models.

Humanized mice as disease models

Despite the fact that mice are the immunologist's favourite animal, approximately 65 million years of evolution separate mice and men. Differences in Toll-like receptor expression patterns between different subsets of immune cells is just one of many examples of 'mice telling lies' (reviewed in ref. 38). Therefore, the use of mice as model organisms for the study of immune responses during infectious diseases carries the risk that important human host–pathogen interactions may be overlooked or misinterpreted. Several approaches to humanize the immune system of mice have been described, including the humanized severe combined immunodeficient (hu-SCID) mice where lymphopenic SCID mice are reconstituted with human haematopoietic cells[39] and more recently, newborn lymphopenic mice reconstituted with $CD34^+$ human cord blood cells.[40,41] Both approaches generate a human haematopoietic compartment in mice. However, the hu-SCID mouse system is stable for only a few weeks. In contrast, human $CD34^+$ cord blood cells transferred into newborn mice can be stable for more than 2 months and can generate adaptive immune responses in the adult mouse. On the other hand, BAC transgenic humanized mice allow stable mouse lines to be established for the study of individual or groups of human genes, and importantly, offer the possibility to humanize non haematopoietic components of the immune system, such as stroma-derived factors (chemokines, type I interferons, adhesion molecules, pathogen entry receptors, stromal receptors for pathogen-associated molecular patterns).

Welstead *et al*. published a BAC-based strategy for a measles virus mouse model.[42] Human CD150 or SLAM (signalling lymphocytic activation molecule) is expressed by activated T cells, B cells, macrophages and dendritic cells, and is used by both wild-type and vaccine strains of measles virus. A BAC containing the endogenous human CD150 gene locus led to expression of the human transgene in activated splenic B and T cells and granulocyte–macrophage colony-stimulating factor-derived dendritic cells, thus recapitulating the expression profile found in humans. CD150 transgenic mice could be infected with MV. However, the infection was transient and the virus load remained lower than in humans, reflecting the involvement of other human-specific molecules in the infection cycle of the virus.

Conclusion

The use of large genomic fragments, cloned as YACs, PACs and BACs, allowed the generation of mice with faithful expression of transgenes under the control of regulatory regions present in a genomic configuration, and minimal interference by the chromatin region flanking the transgenic insertion site. In the post-genomic era, and in the context of systems biology, it will be important to generate mice expressing multiple transgenes or gene clusters. With many strategies available to insert reporters or effectors into specific sites of BACs, it will be possible to identify whole pathways and 'interactomes' *in vivo*, or to generate complex humanized mice. Using BAC technology, we can therefore expect the generation of more physiological mouse models for the study of the murine and human immune systems and human diseases.

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This paper is dedicated to Hermann Wagner on the occasion of his 65th birthday.

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